

POSTN⁺ fibroblast-secreted small extracellular vesicles drive macrophage M2 polarization through BMP4/BMPR2/Smad signaling

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Abstract. Carcinoma-associated fibroblasts (CAFs) exhibit notably heterogeneity and are closely implicated in immune checkpoint blockade (ICB) resistance in head and neck squamous cell carcinoma (HNSCC). However, the specific subtypes and mechanisms involved remain to be elucidated. By analyzing two single-cell RNA sequencing datasets (GSE103322 and GSE139324) and The Cancer Genome Atlas (TCGA)-HNSCC dataset, two distinct fibroblasts subtypes were identified: Perostin (POSTN)⁻ and POSTN⁺ fibroblasts. A comparison with reported markers revealed that extracellular matrix-related markers were highly expressed in POSTN⁺ fibroblasts. In addition, fibroblast activation protein and POSTN expression were positively associated with macrophage infiltration and predicted ICB resistance in TCGA-HNSCC

dataset. Immunogold labeling confirmed the enrichment of POSTN on the membrane surface of CAF-derived small extracellular vesicles (sEVs) and it was indicated that these POSTN⁺ sEVs may promote THP-1-derived macrophages polarization toward the M2 phenotype. Additionally, sEVs derived from CAFs with POSTN knockdown reduced bone morphogenetic protein (BMP) 4 expression in macrophages, thereby inhibiting M2 polarization through the BMP receptor 2/Smad pathway. Collectively, these findings revealed that a POSTN⁺ fibroblasts fosters an immunosuppressive microenvironment via sEV-mediated macrophage polarization, nominating POSTN as a potential therapeutic target to overcome ICB resistance in HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a prevalent malignancy, representing >90% of all head and neck cases (1,2). While patients with early-stage HNSCC generally have a favorable prognosis, those with advanced disease tend to have notably poorer outcomes (3,4). Although immunotherapy, particularly immune checkpoint blockade (ICB) targeting the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) axis, has improved survival in recurrent/metastatic HNSCC (1,5,6), the molecular mechanisms underlying ICB resistance remain poorly understood. Accumulating evidence has implicated the tumor immune microenvironment as a critical determinant (7).

The tumor microenvironment (TME), particularly carcinoma-associated fibroblasts (CAFs), drives immune evasion and therapy resistance (8-10). CAFs influence the infiltration of immunosuppressive cells, promote CD8⁺ T-cell exhaustion, modulate natural killer cell activation and alter macrophage polarization (11). Moreover, CAFs contribute to establishing

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an immunosuppressive TME (12,13). Emerging evidence has indicated that CAFs may contribute to reduced responsiveness to ICB treatment (14,15).

CAFs heterogeneity markedly influences the responses of various types of cancer to ICB. For example, fibroblast activation protein (FAP)⁺ CAFs, subdivided into TGF- β and extracellular matrix (ECM)-driven subclusters, promote resistance via a PD-1/cytotoxic T-lymphocyte associated protein 4-dependent regulatory T cell loop (16). Conversely, Meflin-expressing CAFs enhance ICB efficacy in non-small cell lung cancer by promoting CD4⁺ T-cell infiltration and vascularization (17). Similarly, leucine rich repeat containing 15 (LRRRC15)⁺ CAFs mediate resistance by driving CD8⁺ T-cell dysfunction (18). A multi-cancer analysis further consolidated this paradigm by identifying six conserved pan-CAF subtypes with distinct molecular signatures and roles in anti-PD-1/PD-L1 resistance (19).

Macrophages and fibroblasts are found ubiquitously within all tissue types. Tissue-resident macrophages maintain homeostasis via phagocytosis, orchestrating immune responses and promoting tissue damage repair. Macrophages are mainly classified into M1 and M2 phenotypes, based on their functional differentiation state. Proinflammatory factors (such as TNF- α and IL-6) or bacterial products (for example, lipopolysaccharide) induce M1 polarization, whereas immunoregulatory cytokines (including TGF β and IL-10) promote M2 polarization. M2 macrophages secrete proangiogenic factors, such as VEGF and tissue-remodeling enzymes, including MMPs, to facilitate tumor progression (20,21), whereas M1 macrophages release antiangiogenic factors; for example, IL-12 and CXCL10 and exhibit antitumor effects (22).

Periostin (POSTN), a secreted matricellular protein, promotes tumor progression, chemoresistance and poor prognosis across various types of cancer (23–28). POSTN is highly secreted by CAFs and critically shapes the immune microenvironment. For example, in glioma and ovarian cancer, POSTN recruits M2-type tumor-associated macrophages (TAMs) and is associated with poor survival (29,30). Furthermore, POSTN⁺ CAFs orchestrate immunotherapy resistance by forming a spatial unit with secreted phosphoprotein 1 (SPP1)⁺ macrophages. Through IL-6/STAT3 signaling, this cellular coalition establishes an immunosuppressive niche characterized by T-cell exclusion, thereby driving resistance to ICB (31,32).

Extracellular vesicles (EVs) are a heterogeneous family of lipid bilayer membrane-delimited nano-to micro-sized particles naturally released by all cell types (33,34). EVs comprise prototypic endosome-derived exosomes (endosome-derived vesicles assembled via the fusion of multivesicular bodies and released into the extracellular space), microvesicles (cell membrane-derived submicron-sized particles) and apoptotic bodies (35). Conceptually, EVs transport their cargo to target sites, enabling action over distance (33,35). Size-based EV nomenclature categorizes the vesicles as follows: EVs < 200 nm in diameter are classified as small EVs (sEVs), whereas EVs sized 200–1,000 nm in diameter are classified as large EVs (lEVs) (35). A short POSTN isoform is expressed on the surface of exosomes secreted by human cardiac explant-derived progenitor cells (36). Lysyl oxidase (LOX) is localized on CAF-derived sEV surfaces through its binding to POSTN (37).

However, it remains unclear how POSTN-enriched CAF sEVs regulate macrophage polarization.

In the present study, integration of two HNSCC single-cell RNA sequencing (scRNA-seq) datasets revealed two distinct fibroblasts subtypes: POSTN[−] and POSTN⁺. Furthermore, the co-localization of FAP and POSTN in the stromal compartment of HNSCC tissues was revealed. Notably, FAP and POSTN expression exhibited a markedly positive correlation with macrophage infiltration. Mechanistically, POSTN⁺ CAF-derived sEVs could drive macrophage M2 polarization. Bone morphogenetic protein (BMP) 4 was downregulated in macrophages treated with POSTN-silenced CAF-derived sEVs, further demonstrating that BMP4 promoted M2 polarization through BMP receptor 2 (BMPR2)/Smad signaling. Finally, integrated scRNA-seq and The Cancer Genome Atlas (TCGA)-HNSCC analyses elucidated the role of POSTN in modulating macrophage M2 polarization. The current study demonstrated that the POSTN⁺ fibroblasts may promote an immunosuppressive microenvironment in HNSCC by driving sEV-mediated macrophage M2 polarization, directly implicating POSTN as a potential target for mitigating ICB resistance.

Materials and methods

Data acquisition, quality control and processing. Single-cell RNA sequencing (scRNA-seq) data were obtained from the GSE103322 (38) and GSE139324 (38,39) datasets acquired from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>). GSE103322 dataset was composed of 18 HNSCC patient samples (containing 5,902 single cells). GSE139324 dataset involved 26 paired samples of peripheral blood mononuclear cells (PBMCs) and tumor-infiltrating immune cells from HNSCC patients 18 human papillomavirus (HPV)-negative and eight HPV-positive, six PBMC samples from healthy donors and five tissue-resident immune cell samples from healthy tonsils (totaling 131,224 cells). The two datasets were merged, resulting in a total of 136,206 single cells.

Data processing and analysis were carried out in R (version 4.4.2; <https://www.r-project.org/>) using the Seurat package (version 5.3.1; <https://satijalab.org/seurat/>). Quality control was performed by retaining cells with 300–7,000 detected genes (nFeature_RNA) and less than 10% mitochondrial gene expression (percent.mt). The top 2,000 variable genes were selected for downstream analysis. Nonlinear dimensionality reduction was conducted via Uniform Manifold Approximation and Projection (UMAP), based on 15 principal components and a clustering resolution of 0.5.

Additionally, transcriptomic FPKM data from The Cancer Genome Atlas (TCGA)-HNSCC patients were obtained from cBioPortal (<https://www.cbioportal.org/>). To correct for batch effects across subgroups, Harmony (version 0.1.0; <https://github.com/immunogenomics/harmony>) was applied. Subsequent subgroup re-analysis adhered to the standard Seurat workflow. Immune infiltration levels, assessed via single-sample gene set enrichment analysis (ssGSEA), acquired from the TISIDB database (<http://cis.hku.hk/TISIDB/download.php>).

Cell clustering and annotation. Dimensionality reduction was first performed on the dataset by applying principal component analysis (PCA) to the top 2,000 highly variable

genes. Subsequently, cell clustering was carried out using Seurat's FindClusters function with a resolution of 0.7. Finally, marker genes for the resulting cell subpopulations were identified. All cells annotated based on the conventional markers as previously reported. The following markers were used: Epithelial makers (EpCAM, SFN, KRT14 and KRT5); Fibroblasts markers (FN1, DCN, COL1A1, COL1A2, COL3A1 and COL6A1); T cell markers (CD2, CD3D, CD3E and CD3G); B cell markers (CD19, CD79A, MS4A1, JCHAIN and MZB1); Myeloid markers (CD68, LYZ, CD14, IL3RA, LAMP3, CLEC4C and TPSAB1). Subsequently, the fibroblasts (containing 1,761 single cells) were re-clustered into two subclusters, POSTN⁺ fibroblasts and POSTN⁻ fibroblasts. Myeloid (containing 30,018 single cells) were re-clustered into two subclusters, M1-like macrophage and M2-like macrophage for further analysis.

Bioinformatic analysis of the TCGA database. Gene expression data of TCGA-HNSCC patients (<https://portal.gdc.cancer.gov/projects/TCGA-HNSCC>) were downloaded through the TCGA biolinks R package (version 2.34.1; <https://bioconductor.org/packages/TCGAbiolinks/>). Transcripts per million reads (TPM) were transformed to Log₂(TPM + 1) for further analysis. High and low expression groups were determined according to the median values of POSTN and FAP. The potential response to immune checkpoint blockade ICB was predicted by the tumor immune dysfunction and exclusion (TIDE) algorithm. The expression levels of POSTN and FAP along with immune infiltration levels were quantified using ssGSEA. Based on the ssGSEA enrichment scores of 28 immune signatures, the present study performed unsupervised hierarchical clustering to identify immune-related subtypes. TME was further characterized by calculating the StromalScore, ImmuneScore, ESTIMATEScore using the ESTIMATE algorithm and tumor purity was inferred accordingly. Similarly, molecular subtypes were identified by clustering the entire gene expression dataset. The distinction between POSTN⁺ and POSTN⁻ fibroblasts signatures was visualized using t-distributed stochastic neighbor embedding (t-SNE).

Immunohistochemistry. Patient inclusion and exclusion criteria were established to define a homogeneous cohort for analysis. Inclusion criteria comprised: i) a pathological diagnosis of oral squamous cell carcinoma (OSCC); ii) consecutive enrollment from patients undergoing primary surgical resection at Shanghai Stomatological Hospital; iii) availability of adequately preserved formalin-fixed paraffin-embedded (FFPE) tumor tissues; and iv) availability of complete clinical data. Exclusion criteria were: i) Receipt of neoadjuvant therapy prior to surgery; ii) presence of another simultaneous active malignancy; iii) insufficient or poor-quality FFPE blocks unsuitable for sectioning; and iv) incomplete clinical records.

Based on these criteria, paraffin-embedded tissue sections (4 μm) from 40 patients with oral squamous cell carcinoma (OSCC) obtained from Shanghai Stomatological Hospital who underwent surgical resection were included in this study, with 14 normal oral mucosa samples serving as controls. The cohort comprised 26 male and 14 female patients, with 17 patients aged ≤62 years and 23 patients aged ≥62 years. The mean age was 62 years. Clinical and histopathological characteristics

of the cohort are summarized in Table SIII and all diagnoses were confirmed by hematoxylin and eosin (H&E) staining.

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Immunohistochemical staining was conducted using the SPlink Detection Kit (SP-9000; Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.). Briefly, tissue sections were deparaffinized in xylene, rehydrated through a graded ethanol series and treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. After blocking nonspecific binding with 10% goat serum (cat. no. C0265; Beyotime Biotechnology, Inc.), the sections were incubated overnight at 4°C with primary antibodies against FAP (cat. no. ab314075; 1:200; Abcam) and POSTN (cat. no. ab14041; 1:100; Abcam). Following this, the sections were incubated with biotinylated goat anti-rabbit IgG (cat. no. ab64256; 1:200; Abcam). at room temperature for 20 min and then washed with PBS at room temperature for 10 min. Immunoreactivity was visualized using 3,3'-diaminobenzidine (DAB) as the chromogen detection, followed by hematoxylin counterstaining at room temperature for 5 min. Negative controls were processed similarly, with the primary antibody replaced by PBS.

To ensure analytical reliability, a rigorous quality assessment was implemented. All stained slides were independently evaluated by two certified pathologists blinded to the clinicopathological data. Slides were included in the final analysis only if they met the following pre-defined criteria: i) adequate control staining (positive/negative controls performing as expected); ii) optimal morphological preservation of tissue structure; and iii) low non-specific background interference. Several slides were excluded during this quality control process due to failure to meet these standards.

Stained sections were air-dried and examined under a Leica Thunder DM6B optical microscope (Leica Microsystems GmbH). Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.) was used to measure the integrated optical density and the area of target protein distribution. The mean density per case was determined by averaging values from at least 10 randomly selected fields.

Immunofluorescence. A total of four representative HNSCC tissue samples were used to capture biological heterogeneity while enabling detailed exploratory analysis. Tissue sections from four HNSCC cases were deparaffinized, rehydrated and subjected to heat-induced antigen retrieval in pH 6.0 citrate buffer (cat. no. C1010; Beijing Solarbio Science & Technology Co., Ltd.) at 95°C for 5 min. Subsequently, the sections were washed with PBS at room temperature for 10 min. After blocking nonspecific binding with 10% QuickBlock Blocking Buffer (cat. no. P0220; Beyotime Biotechnology Inc.), the sections were incubated overnight at 4°C with the following primary antibodies: POSTN (cat. no. ab14041; 1:100; Abcam) and CD163 (cat. no. sc-33715; 1:1,000; Santa Cruz Biotechnology, Inc.). The following day, sections were washed with 1X TBST (containing 0.1% Tween-20) at room temperature for 30 min and then incubated at room temperature for 60 min with species-appropriate fluorescent secondary antibodies: Goat Anti-Rabbit IgG AF488 (cat. no. ab150077; 1:200; Abcam) or Goat Anti-Mouse IgG AF594 (cat. no. ab150116;

1:200; Abcam). The sections were washed with 1X TBST at room temperature for 10 min. Nuclei were stained with DAPI (1:3,000; Thermo Fisher Scientific, Inc.) at room temperature for 5 min and then washed with 1X TBST at room temperature for 10 min. Images were acquired using a Leica Thunder DM6B optical microscope (Leica Microsystems GmbH) for subsequent analysis.

Cell culture. The primary cells used in this study—carcinoma-associated fibroblasts (CAFs) derived from human OSCC tissues (CAF-S5 and CAF-S6) and healthy gingival-derived normal fibroblasts (NFs) are identical to those that were isolated and characterized in our previous study (37). CAFs and NFs were maintained in DMEM/F12 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% Fetal bovine serum (FBS; ScienCell Research Laboratories, Inc.). Human immortalized monocytic cell line THP-1 was purchased from iCell Bioscience (CVCL_0006) and grown in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (ScienCell Research Laboratories, Inc.). All cultured cells were supplemented with 100 U/ml penicillin and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a humidified atmosphere with 5% CO₂.

Stable transfection of POSTN. POSTN was silenced by CAFs with concentrated lentivirus particles short hairpin (sh)RNA negative control (shNC; sense: 5'-GTTCTCCGAACGTGTCACGT-3'; anti-sense: 5'-ACGTGACACGTCGGA GAAC-3') and POSTN-targeting shRNA (shPOSTN; sense: 5'-CCCATGGAGAGCCAATTAT-3'; anti-sense: 5'-ATAATTGGCTCTCCATGGG-3'). The lentiviral particles and the shRNA constructs were designed and provided by Shanghai GenePharma Co., Ltd. Specifically, the POSTN-targeting shRNA was cloned into the lentiviral vector pHIV-U6-Scramble-ZsGreen-Puro, and the construct was verified by double enzyme digestion and DNA sequencing.

Human immortalized embryonic kidney cell line 293T (CVCL_0063) was purchased from Shanghai Fuheng Biology and cultured in DMEM High medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (ScienCell Research Laboratories, Inc.), 100 U/ml penicillin, and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a humidified atmosphere with 5% CO₂.

When 293T cells reached 85–90% confluence in 10 cm dishes were co-transfected with 10 µg of the lentiviral shRNA vector (pHIV-U6-Scramble-ZsGreen-Puro), 7.5 µg of the packaging plasmid pSPAX2, and 2.5 µg of envelope plasmid pMD2G using Lipofectamine 3000 (L3000015; Thermo Fisher Scientific, Inc.), following a second-generation system. This combination yielded a plasmid mass ratio of 4:3:1. Viral supernatant was harvested 48 h post-transfection, clarified by centrifugation (3,000 rpm at room temperature for 10 min), and filtered through a 0.45 µm membrane. The viral particles were then concentrated via ultracentrifugation (50,000 x g at 4°C for 2 h) and resuspended in 200 µl of DMEM High medium (Gibco; Thermo Fisher Scientific, Inc.). Subsequently, CAF-S5 and CAF-S6 were incubated overnight with 8 mg/ml Polybrene (cat. no. TR-1003; Sigma-Aldrich; Merck KGaA)

and either control lentiviral particles or those carrying POSTN-targeting shRNA at a multiplicity of infection (MOI) of 100. At 48 h post-transfection, media containing 2 mg/ml puromycin (Sigma-Aldrich; Merck KGaA) were used to select stable clones for two weeks.

PMA-induced THP-1 differentiation into macrophages. The human monocytic leukemia cell line THP-1 was grown in a T25 culture flask supplemented with RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; cat. no. P8139; Sigma-Aldrich; Merck KGaA) for 48 h. Then, they were maintained in fresh RPMI 1640 culture medium for 24 h to stabilize their growth.

Cell treatment. To confirm that BMP4 promotes macrophage polarization toward the M2 phenotype, THP-1 (2 × 10⁶ cells) were seeded in 6-well plates and differentiated into macrophages using 50 ng/ml PMA for 48 h. After stabilization, macrophages were assigned to different groups and then either left unstimulated or stimulated for an additional 48 h with recombinant human BMP4 (50 or 100 ng/ml; cat. no. HY-P7007; MedChemExpress). Macrophages were stained with FITC anti-human CD163 antibody (cat. no. 333617; 1:50; Biolegend Inc.) and FITC anti-human CD206 antibody (cat. no. 321103; 1:50; Biolegend Inc.) at 4°C for 15 min and then centrifugation 1,000 x g at 4°C for 5 min. Pellets were resuspended in 1 ml PBS and analyzed the expression of M2 macrophage polarization biomarkers (CD163 and CD206) was then analyzed by Flow cytometry (NovoCyte 2040R; ACEA Biosciences) equipped with NovoExpress software (version 1.6.3; <https://explore.agilent.com/ACEA-joins-Agilent>). To further to confirm the role of Smad signaling pathway, 10 µM LDN193189 inhibitor (cat. no. HY-12071; MedChemExpress) was added to each well for 48 h during the BMP4 stimulation. Cellular proteins were then harvested and subjected to western blotting to evaluate the expression of markers associated with Smad-related and M2 macrophage polarization.

Isolation, characterization of sEVs and labeling. sEVs isolation methods were performed as previously described (37). EV-depleted FBS was obtained by centrifugation at 100,000 x g at 4°C for 3 h. At 80–90% confluence, CAFs and NFs were washed with PBS three times and cultured in DMEM/F12 medium with 5% EV-depleted FBS for another 72 h. The resulting medium was collected as conditioned medium (CM). Then, CM was differentially centrifuged 500 x g for 10 min, 2,500 x g at 4°C for 20 min, 12,000 x g at 4°C for 30 min, filtered (pore size 0.22 µm), collected by ultracentrifugation at 100,000 x g at 4°C for 70 min. The pellet was diluted in 20 ml PBS and ultracentrifuged at 100,000 x g at 4°C for 70 min. The concentration of sEVs was determined by Nanoflow (N30E; NanoFCM, Co., Ltd.). Purified sEVs in PBS were placed on a formvar carbon-coated 200 mesh grid (cat. no. 200M-Cu; EMCN, Inc.) at room temperature for 20 min, washed twice with PBS and then stained 2% uranyl acetate at room temperature for 60 sec. Analysis of sEVs morphology and structure was performed with a transmission electron microscope (TEM; JEM-2000EX; JEOL, Ltd.) equipped with a CCD camera (Gatan SC1000, Model 832). Images were acquired and analyzed using digital Micrograph software (version 3.6.1; Gatan, Inc.). Positive

and negative marker expression of sEVs were confirmed by western blotting. The particles size and concentration of sEVs were determined by Nanoflow (N30E, NanoFCM). sEVs were labeled with PKH26 (cat. no. PKH26PCL, Sigma-Aldrich; Merck KGaA) according to the company's instructions. Labeled sEVs were re-separated by ultracentrifugation at 100,000 x g at 4°C for 70 min.

OptiPrep density gradient purification. Purification of sEVs was performed using an OptiPrep density gradient, as previously described (40). In brief, 60, 50, 40, 30, 25, 15, 10 and 5% (w/v) iodixanol solutions were prepared by diluting OptiPrep (60% (w/v) (cat. no. D1556, Sigma-Aldrich; Merck KGaA) with 0.25 M sucrose/10 mM Tris, pH 7.5 in Ultra-Clear tubes (cat. no. 344059; Beckman Coulter, Inc.). Purified sEVs were subjected to density gradient ultracentrifugation. The sample was loaded onto a continuous gradient and centrifuged at 100,000 x g at 4°C for 18 h using an Optima XPN-100 ultracentrifuge with a Ti41 rotor (Beckman Coulter, Inc.), resulting in eight collected fractions. Each fraction was diluted in PBS, pelleted by ultracentrifugation at 100,000 x g at 4°C for 70 min in Ultra-Clear tubes and finally washed and resuspended in PBS.

Immunogold labeling of sEVs. Purified sEVs were adsorbed onto formvar carbon-coated grids for 20 min. After three washes with PBS, free aldehyde groups were quenched by incubation with 50 mmol/l glycine. Following nine additional PBS washes, nonspecific sites were blocked with 5% BSA for 1 h. Grids were then incubated overnight at 4°C with primary antibodies (cat. no. CD9/POSTN; 10 µg/ml; Abcam). After incubation at room temperature with a 5 nm colloidal gold-conjugated secondary antibody (cat. no. A31565; 1:50; Thermo Fisher Scientific, Inc.) and a wash with PBS containing 0.1% BSA, samples were fixed at room temperature in 2.5% glutaraldehyde for 15 min. Finally, after thorough deionized water rinsing, grids were counterstained with phosphotungstic acid at room temperature for 5 min and imaged using a JEM-2000EX transmission electron microscope (JEOL, Ltd.).

Uptake of PKH26-labeled sEVs by macrophages. The purified sEVs in 50 µl PBS were fluorescently labeled with 0.4 µl PKH26 Dye (Sigma-Aldrich; Merck KGaA) at room temperature for 20 min. The labeled sEVs were then centrifuged at 100,000 x g at 4°C for 70 min. After discarding the supernatant, equal amounts of sEVs were added to the macrophages at 37°C for 2 h and 6 h, respectively. Macrophages were then fixed in 4% paraformaldehyde at room temperature for 10 min. After centrifugation at 1,000 x g at room temperature for 5 min, the macrophages were resuspended in 1 ml PBS and the percentage of fluorescence was analyzed using a Flow Cytometer (NovoCyte 2040R; ACEA Biosciences).

CAF sEVs treatment for macrophages and flow cytometry analysis. To confirm whether shNC and shPOSTN sEV-derived CAF-S5/-S6 induce macrophage polarization into M1 and M2 macrophage, sEVs (5x10⁹ particles) were added to macrophage (2x10⁶ cells) grown in 6-well plate for 48 h. To test whether CAF-S5/-S6 sEV-POSTN directly induced M2 macrophage polarization, sEVs (5x10⁹ particles) were

incubated overnight 4°C with a POSTN-neutralizing antibody. These pre-treated sEVs were then co-incubated with macrophage (2x10⁶ cells) grown in 6-well plate at 37°C for 48 h. For staining, 100 µl of macrophage (1x10⁶ cells) was incubated with 5 µl of FITC anti-human CD80 antibody (cat. no. 375405; BioLegend, Inc.), FITC anti-human CD86 antibody (cat. no. 374203; BioLegend, Inc.), FITC anti-human CD163 antibody (cat. no. 333617; BioLegend, Inc.) and FITC anti-human CD206 antibody (cat. no. 321103; BioLegend, Inc.) on ice for 15 min and washed twice with PBS. The stained cells resuspended in 1 ml PBS and analyzed on a Flow cytometer (NovoCyte 2040R, ACEA Biosciences) equipped with NovoExpress software (version 1.6.3; <https://explore.agilent.com/ACEA-joins-Agilent>).

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc.) according to the manufacture's protocol. Brief, THP-1-derived macrophages (2x10⁶ cells) treated with PBS (CTRL), shNC sEVs (5x10⁹ particles) and shPOSTN sEVs (5x10⁹ particles) for 48 h. Total RNA was precipitated using isopropanol, washed with 100% ethanol and resuspended in 30 µl of DEPC water. RNA concentration was quantified using a Nanodrop One spectrophotometer (Thermo Fisher Scientific, Inc.). Reverse transcription was performed with the PrimerScript RT reagent Kit (Takara Biotechnology Co., Ltd.) according to the manufacture's protocol, followed by quantitative real-time PCR using the SYBR Premix Ex Taq reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacture's protocol on a LightCycler 480II system (Roche Diagnostics) with the following cycling protocol: Initial denaturation at 95°C for 15 min; followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 56-60°C for 20 sec and extension at 72°C for 32 sec. GAPDH was used as an internal control. The data for each sample were semi-quantitatively analyzed using the 2^{-ΔΔC_q} method (41). The primers for CD68, CD80, CD86, CD163, CD206, BMP4, TNF-α, IL-6, TGF-β, IL-10 and GAPDH were purchased from Sangon Biotech Co., Ltd. Primer sequences were as following: CD68 (forward): 5'-AGCCACAAAACCACCACTCA-3', (reverse): 5'-CTA GTGGTGGCAGGACTGTG-3'; CD80 (forward): 5'-GCA GGGAACATCACCATCCA-3', (reverse): 5'-CACTTCCTT GGTCACGTGGA-3'; CD86 (forward): 5'-GGAAGAAGA AGAAGCGGCCT-3', (reverse): 5'-CGCTGGGCTTCATCA GATCT-3'; CD163 (forward): 5'-GCGGGAGAGTGGGAAG TGAAA-3'; (reverse): 5'-ACCTGCACTGGAATTAGCCC-3'; CD206 (MRC1) (forward): 5'-AGGATGGGTACTGGGCAG AT-3'; (reverse): 5'-CTGGACCTTGGCTTCGTGAT-3'; BMP4 (forward): 5'-GGAGGAGGAGGAAGAGCA GA-3', (reverse): 5'-TTCTTCGTGTTGGAAGCTCC-3'; TNF-α (forward): 5'-CTTCCAGCTGGAGAAGGGTG-3', (reverse): 5'-CCC AAAGTAGACCTGCCAG-3'; IL-6 (forward): 5'-AGTGAG GAACAAGCCAGAGC-3', (reverse): 5'-GGTCAGGGGTGG TTATTGCA-3'; TGF-β1 (forward): 5'-GCCCTGGACACC AACTATT-3'; (reverse): 5'-AGGCTCCAAATGTAGGGG-3'; IL-10 (forward): 5'-AAGACCCAGACATCA AGGCG-3', (reverse): 5'-AGGCATTCTTCACCTGCTCC-3'; GAPDH (forward): 5'-GTGAAGGTCGGAGTCAACG-3', (reverse): 5'-TGAGGTCAATGAAGGGGTC-3'. Each experiment was repeated at least three times.

Western blotting. Total protein was extracted with RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) supplemented with protease (cat. no. HY-K0010; MedChemExpress) and phosphatase inhibitors (cat. no. HY-K0021; MedChemExpress) at 4°C for 30 min. The lysates were then subjected to protein concentration measurement with a BCA kit (cat. no. P0009; Beyotime Biotechnology). Proteins (20 µg per lane) were separated by 4-20% SDS-PAGE and subsequently transferred to a PVDF membrane (MilliporeSigma). The membrane was then blocked with 5% fat-free milk for 1 h at room temperature to prevent non-specific binding, followed by an overnight incubation with primary antibodies at 4°C. The primary antibodies included POSTN antibody (cat. no. ab14041; 1:500; Abcam), CD9 (cat. no. ab236630; 1:1,000; Abcam), CD81 (cat. no. ab79559; 1:1,000; Abcam), Calnexin (10427-2-AP; 1:1,000, Proteintech, Wuhan, China), CD80 (cat. no. ab134120; 1:1,000; Abcam), CD86 (cat. no. abs115477; 1:1,000; Absin Bioscience), CD163 (sc-33715; 1:1,000, Santa Cruz Biotechnology), CD206 (cat. no. ab64693; 1:1,000; Abcam), BMPR2 (cat. no. abs147034; 1:1,000; Absin Bioscience), Phospho-Smad1 (Ser463/465)/Smad5(Ser463/465)/Smad9(Ser465/467) (cat. no. 13820; 1:1,000; Cell Signaling Technology, Inc.), Smad5 (12534; 1:1,000; Cell Signaling Technology, Inc.), Smad9 (cat. no. abs131190; 1:1,000; Absin Bioscience) and GAPDH (cat. no. 10494-1-AP; 1:5,000; Proteintech Group, Inc.), followed by incubation with a HRP-conjugated Goat anti-Rabbit IgG (H+L) as the secondary antibody (cat. no. SA00001-2; 1:3,000; Proteintech Group, Inc.) for 2 h. After washing the membrane three times with 1X TBST, the protein bands were visualized using an ECL detection system. Amersham ImageQuant 800 (Cytiva) and the intensity of the band was quantified using ImageJ software (version 1.54k; National Institutes of Health).

Enzyme-linked immunosorbent assay (ELISA). To confirm the presence of POSTN on the surface of CAFsEVs, Human POSTN ELISA kits was purchased (cat. no. H2452c, Elabscience). To assess the content of BMP4 on the surface of sEV-derived CAF-S5/-S6 transfected with shNC and shPOSTN, Human BMP4 ELISA kits was purchased (cat. no. CSB-E17298h; Cusabio Technology, LLC). Serially diluted CAF-derived sEVs were added to each well (100 µl/well) and incubated for 90 min. Subsequently, biotinylated POSTN and BMP4 antibody was introduced and incubated at 37°C for 1 h, followed by sequential additions of horseradish peroxidase-conjugated streptavidin (100 µl, 37°C for 30 min) and 3,3',5,5'-tetramethylbenzidine substrate (37°C for 15 min). The reaction was terminated with 50 µl of stop solution and absorbance at 450 nm was measured using a Thermo Fisher Scientific, Inc. microplate reader.

For POSTN blocking on the sEV surface, CAF sEVs were incubated overnight at 4°C with a POSTN-blocking antibody (10 µg/ml in 100 µl PBS; Abcam). Non-bound antibodies were removed by washing with 20 ml PBS and ultracentrifugation at 100,000 × g at 4°C for 70 min. All experiments were performed in at least triplicate.

RNA sequencing analysis. Total RNA was extracted from macrophages treated with CAF sEV-derived shNC and shPOSTN for 48 h using TRIzol® Reagent (Thermo Fisher

Scientific, Inc.) per the manufacturer's protocol. RNA integrity was assessed by Agilent 5300 Bioanalyser and concentration was measured using a Nanodrop ND-2000. High-quality RNA samples were used for library preparation. Subsequently, RNA purification, reverse transcription, library construction and sequencing were conducted by Majorbio Biotechnology on an Illumina NovaSeq/HiSeq Xten (Illumina, Inc.) platform with NovaSeq reagents kits (Illumina, Inc.) according to standard protocol. Raw paired-end reads were processed with Fastp (version 1.0.1; <https://github.com/OpenGene/fastp>) for adapter trimming and quality control. HISAT2 was used to align the cleaned reads to the reference genome and transcript assembly was performed with StringTie (version 3.0.3; <https://ccb.jhu.edu/software/stringtie>). Gene expression was quantified in TPM and differential expression analysis was carried out to identify differentially expressed genes (DEGs) between comparative groups. RSEM was used to quantify gene abundances. Essentially, differential expression analysis was performed using the DEGseq (42). Functional-enrichment analysis including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed to identify which DEGs were significantly enriched in GO terms and metabolic pathways at Bonferroni-corrected P-value ≤ 0.05 compared with the whole-transcriptome background. GO functional enrichment and KEGG pathway analysis were carried out by Goatools and KOBAS (43), respectively.

Statistical analysis. Single-cell sequencing was performed using RStudio software (version 4.1.1). Data distributions were visualized via histograms generated in R software. Categorical variables were compared using the Chi-squared test. For continuous variables, comparisons between two groups used the unpaired two-tailed Student's t-test or the Wilcoxon rank-sum test. Comparisons among three or more groups were performed using one-way ANOVA test followed by Tukey's post hoc test. Inter-variable relationships were examined using Spearman's rank correlation. Statistical analysis and quantification of western blotting bands were performed using GraphPad prism software (version 7.0; Dotmatics) and ImageJ (version 1.6.0; National Institutes of Health). Results are expressed as mean ± SEM from a minimum of three independent experiments. P < 0.05 was considered to indicate a statistically significant difference.

Results

POSTN⁺FAP⁺ fibroblasts are associated with a poor ICB response in HNSCC. An integrated analysis of the HNSCC single-cell profiling data was conducted using sequencing datasets (Fig. 1A), revealing five clusters that were annotated via Seurat clustering according to specific markers. The gene lists associated with cluster annotation markers are included in Table SI. Existing studies have revealed that POSTN is markedly elevated in tumor-derived fibroblasts compared with in normal tissues (37,44,45). The violinplot demonstrated that POSTN was mainly expressed in the fibroblasts cluster (Fig. 1B). Subsequently, the fibroblasts were further re-clustered into nine clusters (Fig. 1C) and it was revealed that POSTN was expressed in subpopulations 1, 4 and 5, with its expression was predominantly enriched in subpopulation 1 (Fig. 1D).

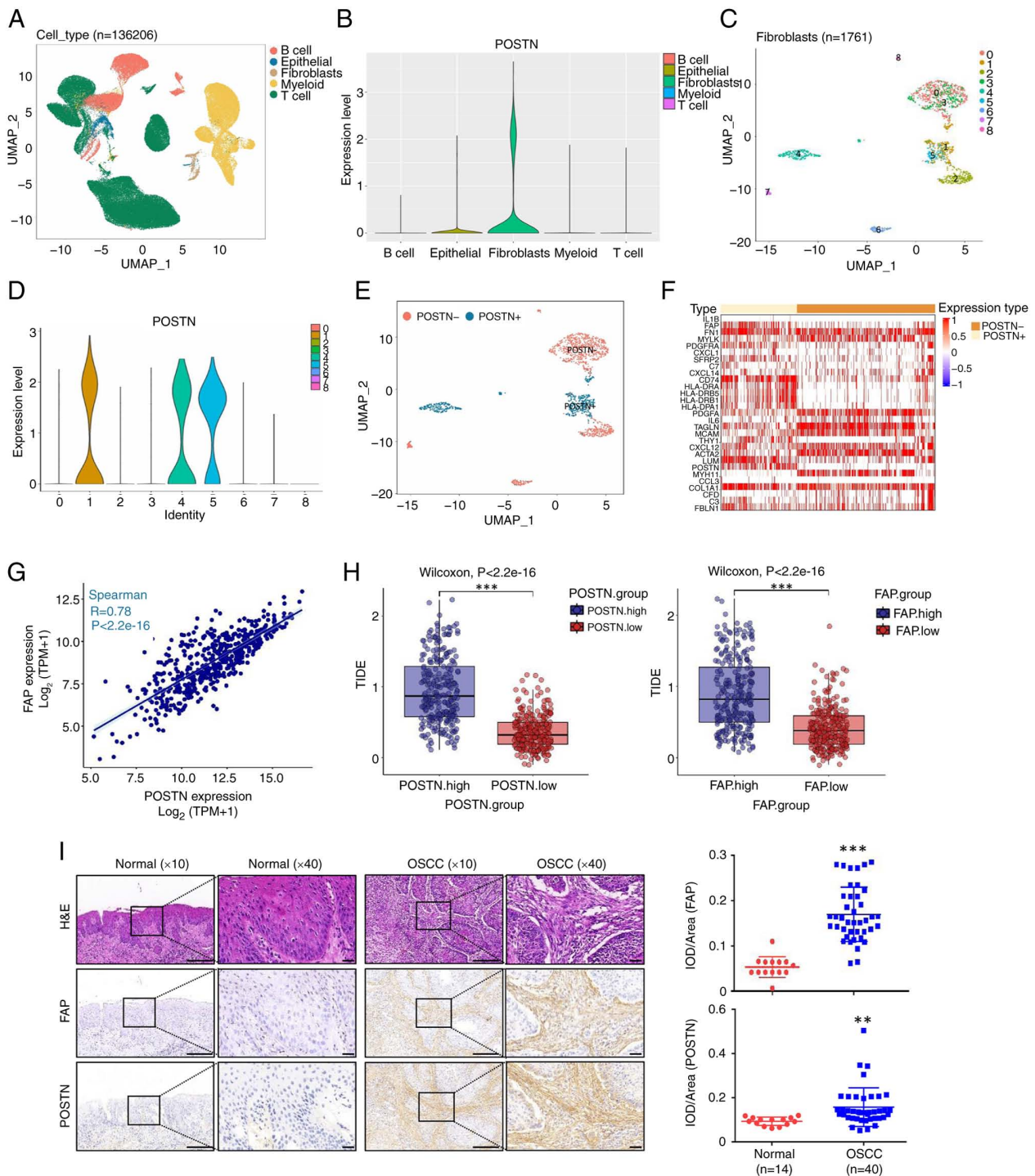


Figure 1. POSTN⁺FAP⁺ fibroblasts contributed to the resistance against ICB in HNSCC. (A) The datasets of GSE103322 and GSE139324 were integrated for further analysis. A total of five clusters were identified previously based on reported biomarkers, including B cell, epithelial, fibroblasts, myeloid and T cells. (B) Vlnplots demonstrated the expression levels of POSTN across five clusters. (C) Fibroblasts re-clustered into nine clusters. (D) Vlnplots visualized the expression levels of POSTN in the nine clusters. (E) POSTN⁻ fibroblasts and POSTN⁺ fibroblasts were annotated based on POSTN expression levels in fibroblasts subtypes. (F) Heatmap displayed the expression levels of cell-type specific biomarkers (myCAF, iCAF, apCAF and eCAF) in POSTN⁻ fibroblasts and POSTN⁺ fibroblasts. (G) POSTN showed a significant correlation with FAP in the TCGA-HNSCC ($^{***}P<0.001$; Spearman's rank correlation). (H) High expression of POSTN and FAP was associated with elevated TIDE scores. Blue and red bars representing higher and lower expression levels, respectively. (I) H&E and IHC analysis of FAP and POSTN expression in OSCC cases (n=40) and normal controls (n=14). Scale bar, 100 and 20 μ m. Right: IOD/Area of FAP and POSTN in the normal and OSCC tissues were quantified. Statistical significance was determined by unpaired two-tailed Student's t-test, $^{**}P<0.01$; $^{***}P<0.001$. Error bars represent the mean $\pm$ SEM. POSTN, perostin; FAP, fibroblast activation protein; ICB, immune checkpoint blockade; HNSCC, head and neck squamous cell carcinoma; TIDE, tumor immune dysfunction and exclusion; H&E, hematoxylin and eosin; IHC, immunohistochemical; OSCC, oral squamous cell carcinoma; IOD, integrated option density.

To determine whether POSTN was expressed in M1/M2 macrophages, the myeloid cluster was further re-clustered

into 14 subtypes. Low POSTN expression was detected in five of these subpopulations and it was negligible in the others

(Fig. S1A). Based on established biomarkers (Table SII), these subpopulations were categorized into M1-like and M2-like macrophages (Fig. S1B). Notably, low expression of POSTN was present in M1-like macrophages and it was barely detectable in M2-like macrophages (Fig. S1C). Based on UMAP clustering analysis, the fibroblast subpopulations were categorized into POSTN⁻ and POSTN⁺ fibroblasts (Fig. 1E). Heatmap analysis revealed that myofibroblastic CAF (myCAF)-related proteins (ACTA2, MYH11, TAGLN, PDGFA, MCAM and MYLK) and inflammatory CAF (iCAF)-related proteins (IL6, CXCL12, C3 and CFD) were predominantly expressed in POSTN⁻ fibroblasts. By contrast, ECM CAF (eCAF)-related proteins (FAP, FN1, SFRP2, THY1, COL1A1, FBLN1 and LUM) and antigen presenting CAF (apCAF)-related proteins (CD74, HLA-DRA, HLA-DRB5, HLA-DRB1 and HLA-DPA1) were predominantly expressed in POSTN⁺ fibroblasts (Fig. 1F). Vnplots further illustrated the expression levels of eCAF and apCAF-associated proteins in POSTN⁺ fibroblasts and POSTN⁻ fibroblasts (Fig. S1D). Spearman's correlation analysis revealed a significant correlation between the expression levels of POSTN and FAP (Fig. 1G). Moreover, patients with POSTN^{high} and FAP^{high} HNSCC exhibited a high poor response to ICB (Fig. 1H). The clinical parameters of 40 OSCC cases and 14 normal controls are listed in Table SIII. H&E and immunohistochemical staining identified higher expression levels of FAP and POSTN in the stroma of OSCC cases compared with those in the normal control samples (Fig. 1I). These findings indicated that POSTN⁺FAP⁺ fibroblasts are localized in the stromal region and associated with a poor response to ICB in patients with HNSCC.

Association of POSTN⁺FAP⁺ fibroblasts with macrophage infiltration and contributing to an immunosuppressive micro-environment in HNSCC. Recent studies have provided evidence for intercellular communication between POSTN⁺FAP⁺ fibroblasts and macrophages (46–48). Additionally, the macrophage infiltration rate is a critical factor in responses to ICB. The ssGSEA algorithm (Fig. 2A) demonstrated that the expression levels of POSTN and FAP were markedly associated with the immune infiltration of macrophages in TCGA-HNSCC. The expression levels of POSTN and FAP exhibited strong positive correlations with markers of M1 (CD80 and CD86) and M2 (CD163 and CD206) macrophages, respectively. However, the correlations with the M2 macrophage markers (CD163, $P < 2.2 \times 10^{-16}$; CD206, $P < 2.2 \times 10^{-16}$) were markedly stronger (Figs. 2B, S2A and B). Immunofluorescence staining revealed the co-expression of POSTN and CD163 in the stromal area in HNSCC cases (Fig. 2C). To delineate the role of POSTN⁺ fibroblasts in the TME, we analyzed 28 immune-related signatures, two distinct immunity subtypes were identified through unsupervised hierarchical clustering: high and low-immunity subtypes (Fig. 2D). The high-immunity subtype was characterized by high stromal, immune, ESTIMATE and POSTN scores coupled with low tumor purity (Fig. 2E). Then, two molecular subtypes of HNSCC-TCGA were identified through unsupervised hierarchical clustering (Fig. S2C and D). Further analysis of the TME revealed that POSTN⁺ fibroblasts were characterized by high stromal, ESTIMATE, immune and POSTN scores, as well as low tumor purity (Fig. S2E). Interestingly, M0 macrophages, M2 macrophages and CD4⁺ T memory cells

also highly infiltrated the POSTN⁺ fibroblasts, highlighting the strong positive correlation between the POSTN⁺ fibroblasts and M2 macrophages in HNSCC (Fig. 2F). Collectively, these findings strongly suggest that POSTN⁺ fibroblasts are associated with an immunosuppressive TME in HNSCC, potentially through their strong correlation with and spatial proximity to M2 macrophages.

POSTN is present on the surface of CAF sEVs. To further confirm the expression levels of POSTN in CAFs and their sEVs, human primary OSCC-derived CAF-S5/-S6 and healthy gingival-derived NFs were successfully extracted and validated, as previously described (37). The sEVs were isolated from the respective CM of CAF-S5/-S6 and NFs using differential ultracentrifugation (Fig. 3A) and they exhibited characteristic exosomal morphology in TEM images (Fig. 3B). These sEVs tested positive for exosomal markers, including CD9, CD81 and ALIX and tested negative for Calnexin (Fig. 3C). The size distribution and particle concentration of sEVs were examined using a Nanoflow cytometer, revealing an mean size of ~100 nm and a concentration of $\sim 1 \times 10^{10}$ sEVs/ml of CM (Fig. 3D). The expression levels of POSTN in CAF-S5/-S6 and their sEVs were markedly higher than those in NFs and their sEVs (Fig. 3E). Furthermore, density gradient fractionation analysis revealed the presence of POSTN on CAF-derived sEVs (Fig. 3F). Immunogold labeling confirmed the presence of POSTN on the surface of CAF-S5/-S6 sEVs (Fig. 3G). ELISA further demonstrated that the level of sEV-POSTN from these cells increased in an sEV dose-dependent manner (Fig. 3H), a signal that was markedly reduced by a POSTN-blocking antibody (Fig. 3H). Taken together, these findings identified POSTN as a surface component of CAF-S5/-S6 sEVs.

CAF-derived sEV-POSTN induces M2 polarization of THP-1-derived macrophages. To determine whether sEV-POSTN can induce macrophage polarization, CAF-S5/-S6 stably transfected with shNC and shPOSTN were established and sEVs were isolated by differential ultracentrifugation. The expression of POSTN in CAF-S5/-S6 and their sEVs was markedly reduced by shPOSTN compared with that in the shNC group (Fig. 4A). The mean sizes of sEVs derived from shNC/shPOSTN-transfected CAF-S5/-S6 were ~95 and 80 nm, respectively and their particles concentration were $\sim 1.9 \times 10^{10}$ and 2.7×10^{10} sEV/1 ml CM, respectively (Fig. 4B). Human THP-1 cells were incubated with PMA (50 ng/ml) for 48 h to induce differentiation into M0 macrophages, which exhibited the corresponding adherent morphology (Fig. 4C) and highly expressed the macrophage marker CD68 ($P < 0.001$; Fig. 4D). Flow cytometry revealed that macrophages exhibited no significant difference in their uptake of PKH26-labeled sEVs derived from shNC/shPOSTN-transfected CAF-S5/-S6 (Fig. 4E). RT-qPCR assay revealed that the M2 macrophage markers CD163 and CD206 were markedly downregulated, whereas the M1 macrophage markers CD80 and CD86 remained unchanged, after co-incubation with sEVs derived from shPOSTN-transfected CAF-S5/-S6 compared with those from sEVs derived from shNC-transfected CAF-S5/-S6 ($P < 0.01$; Fig. 4F). Flow cytometry confirmed that the expression levels of M2 macrophage markers (CD163 and CD206)

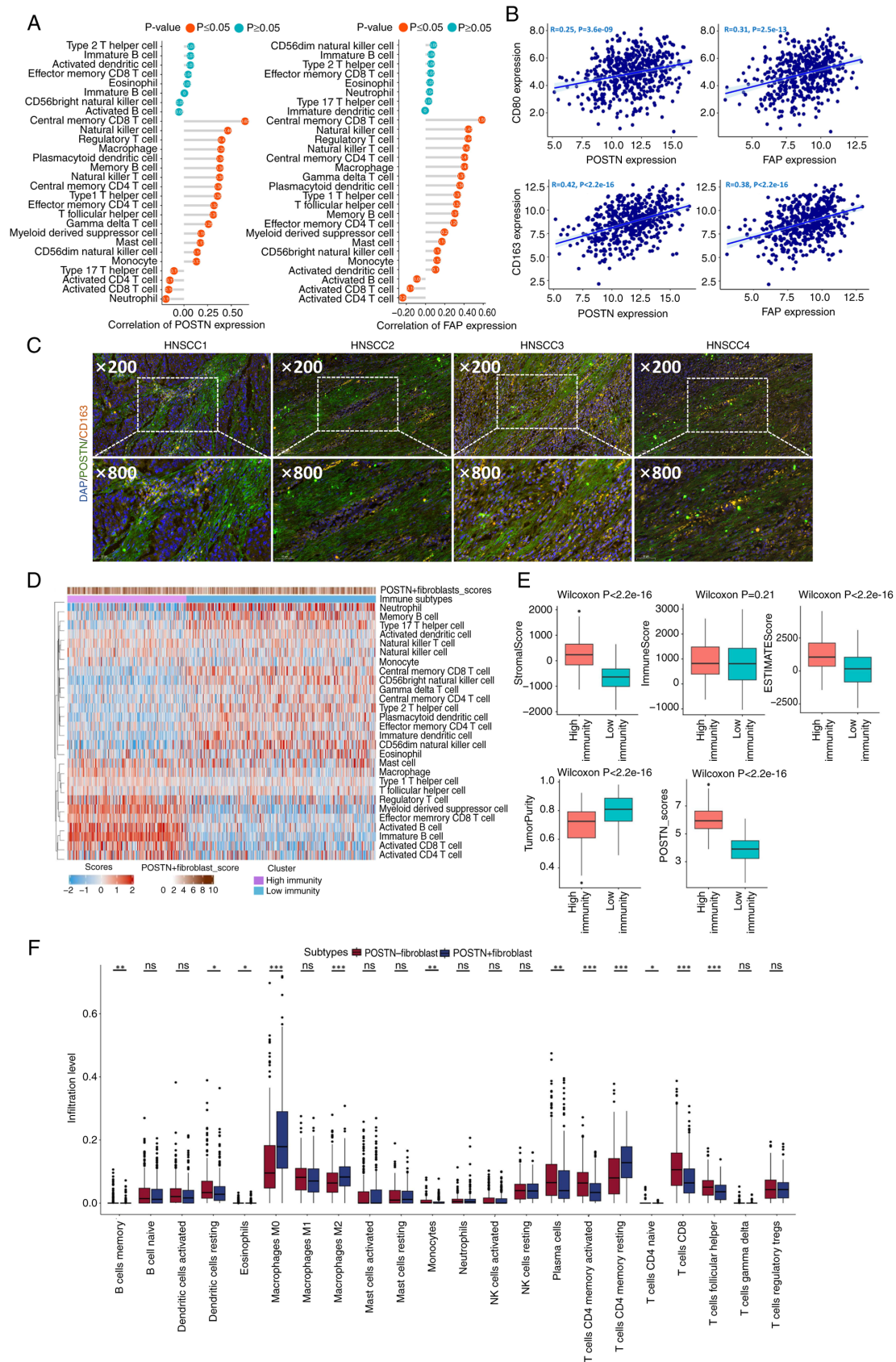


Figure 2. Association of POSTN⁺FAP⁺ fibroblasts with the immune landscape in HNSCC. (A) POSTN and FAP expression associated with the level of immune cell infiltration in the TCGA-HNSCC (Spearman's rank correlation). (B) A significant positive correlation was observed between the expression of POSTN/FAP and CD80/CD163 in the TCGA-HNSCC ($^{***}P < 0.001$; Spearman's rank correlation). (C) Co-expression of POSTN (green) and CD163 (orange) in HNSCC cases (n=4). Top: images of HNSCC tissues (Scale bar, 100 μ m). Bottom: images of HNSCC tissues (Scale bar, 50 μ m). (D) High and low immunity subtypes were identified by unsupervised hierarchical clustering. (E) The distribution of stromal, immune, ESTIMATE, TumorPurity and POSTN scores in high and low immunity subtypes. (F) The infiltration abundances of 22 immune cells across POSTN⁻/POSTN⁺ fibroblasts. Statistical significance was determined by unpaired two-tailed Student's t-test, ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Error bars represent the mean $\pm$ SEM. POSTN, perostin; FAP, fibroblast activation protein; TCGA, The Cancer Genome Atlas; HNSCC, head and neck squamous cell carcinoma.

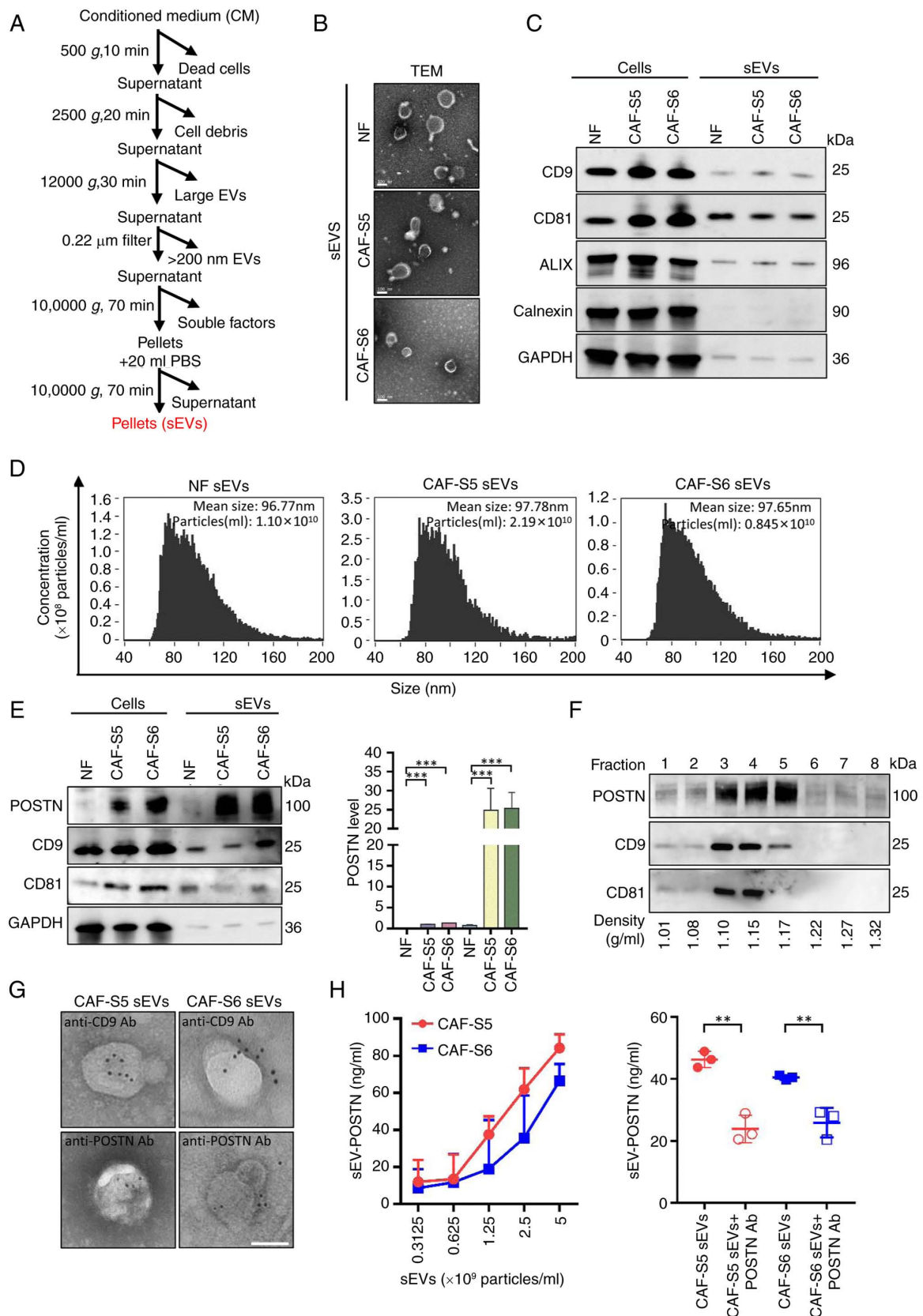


Figure 3. POSTN is located on the surface of sEV-derived from CAFs. (A) Flowchart of sEVs isolation process. (B) The morphology and structure of sEVs examined by TEM. Scale bar, 100 nm. (C) The expression of biomarkers of sEVs were examined by western blotting. CD9, CD81 and ALIX were used as positive biomarkers for sEVs, while Calnexin was used as a negative biomarker. GAPDH was used as internal control. (D) Nanoflow cytometer examined the particles concentration and mean size distribution of sEVs. (E) The expression levels of POSTN in CAFs and their sEVs were measured. CD9 and CD81 were used as positive markers of sEVs (n=3 per group). GAPDH was used as an internal control. (F) Western blotting analysis was performed the expression levels of POSTN in density gradient fractionation of sEV-derived CAF-S6. CD9 and CD81 were used as positive markers of sEVs. (G) TEM images of immunogold-labeled POSTN on CAF-S5/-S6 sEVs (Scale bar, 100 nm). (H) ELISA examination of POSTN in CAF-S5/-S6 sEVs (n=3 per group). Statistical significance was determined by unpaired two-tailed Student's t-test, **P<0.01; ***P<0.001. Error bars represent the mean $\pm$ SEM. For source data for blotting assays, see Fig. S5. POSTN, perostin; sEV, small extracellular vesicles; CAFs, carcinoma-associated fibroblasts; TEM, transmission electron microscopy.

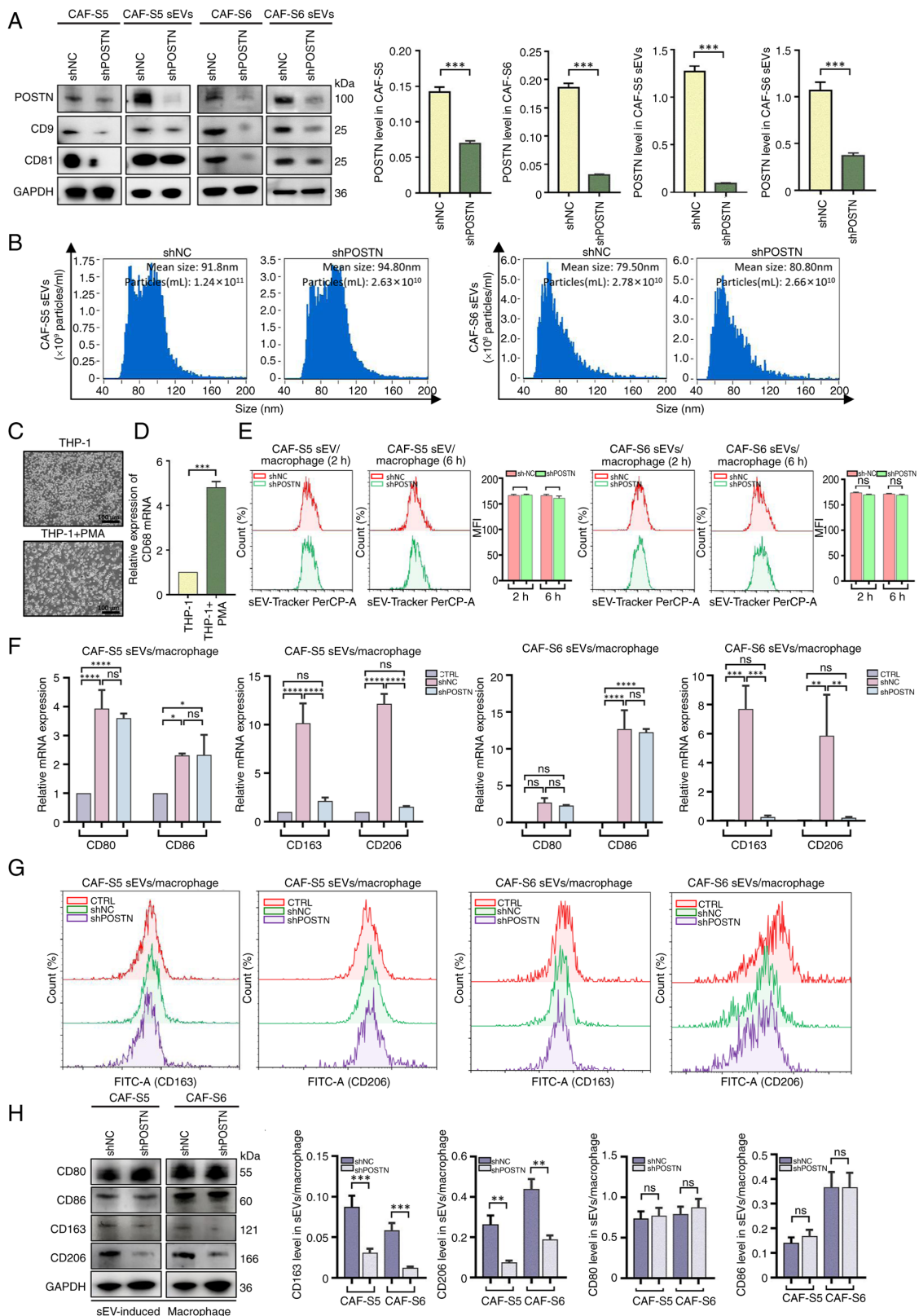


Figure 4. sEV-derived CAFs induced macrophage M2 polarization. (A) The expression levels of POSTN in CAF-S5/-S6 and their sEVs transfected with shNC and shPOSTN were examined by western blotting (n=3 per group). CD9 and CD81 were used as positive markers of sEVs. Left: Representative images. Right: Quantitative analysis of POSTN in different groups. (B) Particles concentration and size distribution of sEV-derived CAF-S5/-S6 transfected with shNC and shPOSTN were analyzed by Nanoflow cytometer. (C) Representative image of macrophages derived from THP-1 treated with PMA (50 ng/ml; scale bar, 100 μ m). (D) The mRNA expression of CD68 was analyzed by RT-qPCR. (E) Internalization of PKH26-labeled sEV-derived CAF-S5/-S6 transfected with shNC and shPOSTN by macrophages was examined by flow cytometer at 2 and 6 h after incubation (n=3 per group). Quantitative analysis of the MFI of PKH26-labeled sEVs internalized by macrophages for 2 and 6 h, respectively. The mRNA expression of CD80, CD86, CD163 and CD206 expression in macrophages were treated with sEV-derived CAF-S5/-S6 transfected with shNC and shPOSTN were analyzed by (F) RT-qPCR, (G) flow cytometry and (H) western blotting. Statistical significance was determined using a one-way ANOVA test. ns, not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Error bars represent the mean $\pm$ SEM. For source data for blotting assays, see Fig. S5. sEV, small extracellular vesicles; CAFs, carcinoma-associated fibroblasts; POSTN, perostin; sh, short hairpin; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; MFI, mean fluorescence intensity.

were markedly reduced in macrophages treated with sEVs derived from POSTN-knockdown CAF-S5/-S6 (Fig. 4G). Furthermore, the expression levels of M2 macrophage markers (CD163 and CD206) were markedly downregulated in macrophages treated with sEVs derived from shPOSTN-transfected CAF-S5/-S6 compared with those treated with sEVs derived from shNC-transfected CAF-S5/S6 (Fig. 4H). However, the expression of M1 macrophage markers (CD80 and CD86) showed no significant difference in macrophages treated with sEVs derived from shNC/shPOSTN-transfected CAF-S5/-S6 (Fig. 4H).

RT-qPCR and western blotting analysis of macrophages also showed that the expression levels of M2 macrophage markers (CD163 and CD206) were markedly downregulated in macrophages treated with CAF-S5/-S6 sEVs pre-incubated with a POSTN-blocking antibody, compared with those treated with CAF-S5/-S6 sEVs (Fig. S3A and B). Flow cytometry confirmed that CAF-S5/-S6 sEVs pre-incubated with a POSTN-blocking antibody markedly reduced the expression levels of M2 macrophage markers (CD163 and CD206) (Fig. S3C and D). These results demonstrated that sEVs secreted by POSTN-knockdown CAF-S5/-S6 may inhibit M2 macrophage polarization.

BMP4 drives macrophage M2 polarization by activating BMPR2/Smad signaling. To further investigate the mechanism by which POSTN regulates THP-1-derived macrophages M2 polarization, RNA sequencing was performed on THP-1-derived macrophages stimulated with sEVs derived from CAF-S6 transfected with shPOSTN and shNC. PCA was employed to assess transcriptomics profiles, revealing a clear distinction between macrophages treated with shPOSTN sEVs and CTRL or shNC sEVs (Fig. 5A). A Venn diagram revealed 15 shared genes and 242 unique genes between macrophages treated with shPOSTN sEVs and shNC sEVs (Fig. 5B). Furthermore, a heatmap showed significant upregulation and downregulation of genes in macrophages stimulated with sEVs derived from CAF-S6 transfected with shPOSTN compared with those treated with sEVs derived from CAF-S6 transfected with shNC (Fig. 5C). A volcano plot identified 443 DEGs, including 262 upregulated and 181 downregulated genes, in macrophages treated with shPOSTN sEVs compared with those treated with shNC sEVs (Fig. 5D). Among the top DEGs, BMP4 was identified as a critical growth factor that fosters a pro-tumoral immune environment by polarizing macrophages toward an M2-like phenotype, thereby promoting tumor growth (49).

GSEA revealed that inflammatory response was upregulated in the macrophages treated with shPOSTN sEVs compared with in those treated with shNC sEVs (Fig. 5E). GO analysis revealed that 20 biological processes were markedly altered in macrophages treated with shPOSTN sEVs compared with shNC sEVs, with notable changes in 'cellular anatomical entity', 'binding' and 'cellular processes' (Fig. 5F). Additionally, KEGG pathway analysis revealed significant enrichment of pathways, such as the 'cytokine-cytokine receptor interaction' and 'viral protein-cytokine receptor interaction', which are closely associated with the inflammatory response during macrophage M2 polarization (50,51) (Fig. 5G). The levels of BMP4 in sEV-derived CAF-S5/-S6

transfected with shNC were higher than those in the shPOSTN group (Fig. S4), average BMP4 level was 2.6 pg/ml in the shNC group and 1.9 pg/ml in the shPOSTN group. Furthermore, RT-qPCR analysis showed markedly higher levels of BMP4 in macrophages treated with sEVs derived from shNC-transfected CAF-S5/-S6 compared with in those treated with sEVs derived from shPOSTN-transfected CAF-S5/-S6 (Fig. 5H). Moreover, the mRNA expression levels of pro-inflammatory factors (TNF- α and IL-6) were markedly downregulated in macrophages with 50 and 100 ng/ml of BMP4 (Fig. 5I). By contrast, the mRNA expression levels of anti-inflammatory factors (TGF- β and IL-10) were markedly upregulated in macrophages with 50 and 100 ng/ml of BMP4 (Fig. 5I). In addition, flow cytometry showed BMP4-induced upregulation of M2 macrophage markers CD163 and CD206 (Fig. 5J). Moreover, the levels of BMPR2 and phospho-Smad1/5/9 were increased in macrophages treated with 100 ng/ml BMP4 for 48 h (Fig. 5K). To assess the role of Smad signaling in BMP4-induced M2 polarization, the BMP type I receptor was inhibited with LDN193189. This treatment suppressed BMP4-triggered Smad1/5/9 phosphorylation and the expression of CD163/CD206 (Fig. 5L). These findings suggested that POSTN-deficient CAF sEVs may decrease BMP4 secretion, which otherwise activates the BMPR2/Smad signaling to drive macrophage M2 polarization, which could offer a therapeutic strategy for targeting TAMs.

Discussion

Researchers have increasingly favored employing scRNA-seq technology to investigate the heterogeneous characteristics of CAFs in solid tumors (52,53). The analytical outcomes of scRNA-seq are subject to notable variability depending on the specific experimental methodology, with each approach exhibiting unique advantages and technical constraints (54). Despite its technical limitations, such as the loss of spatial information and under-representation of certain cell types due to isolation challenges (55), scRNA-seq has been instrumental in identifying distinct CAF subsets, including myCAF, iCAF and apCAF. These subsets possess unique gene expression profiles and functional properties, influencing tumor progression and immune responses through various mechanisms, such as the secretion of cytokines, chemokines and ECM components (56-58). Although ICB targeting PD-1/PD-L1 has improved survival in recurrent or metastatic HNSCC (59,60), its underlying mechanisms require further elucidation. Notably, differences in ICB efficacy associated with CAF subgroups have been reported across diverse solid tumors. Among these, POSTN⁺ fibroblasts are characterized by high α -smooth muscle actin (α SMA) expression and serve crucial roles in ECM regulation and tissue architecture maintenance (61,62). Previous studies have revealed that POSTN⁺ fibroblasts exhibited characteristics of myCAF, as they expressed ECM-associated signature genes (POSTN, MMP14 and MMP11) (31,63,64).

To comprehensively characterize the tumor immune microenvironment in HNSCC, the present study integrated an PBMC-derived scRNA-seq dataset (GSE139324) alongside primary tumor data. This approach was necessitated by the limited availability of HNSCC-specific scRNA-seq resources

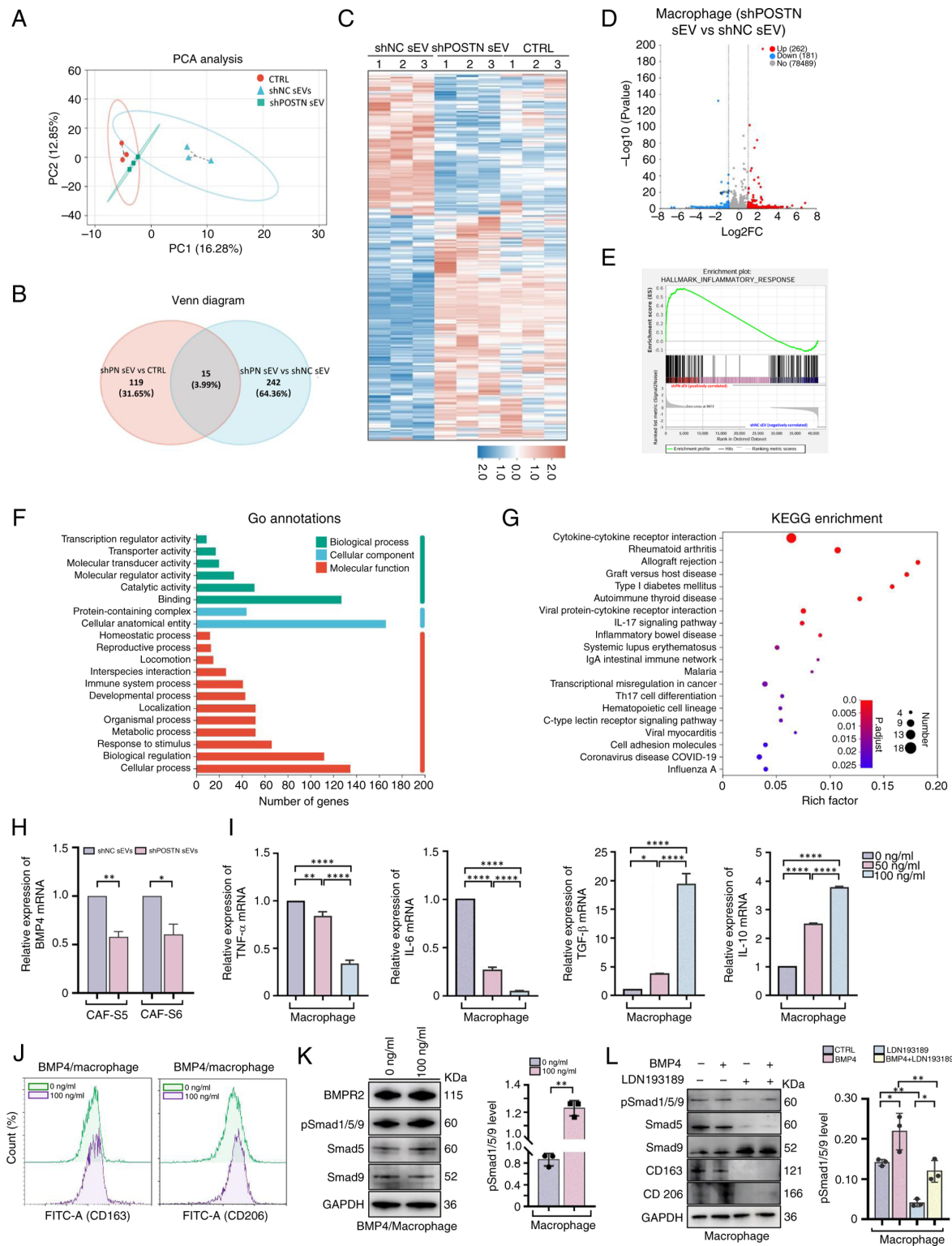


Figure 5. BMP4 activates BMPR2/Smad signaling to induce macrophage M2 polarization. (A) PCA analysis of macrophages were treated with sEV-derived CAF-S6 transfected with shNC and shPOSTN in three replicate times. (B) Venn-diagram of DEGs between shPOSTN sEV vs. CTRL and shPOSTN sEVs vs. shNC sEVs. (C) Heatmap showing the top DEGs in each group. (D) Volcano plot showing BMP4 downregulation in macrophages treated with shPOSTN sEVs vs. shNC sEVs. (E) GSEA analysis of hallmark pathways in macrophages treated with sEV-derived CAF-S6 transfected with either shPOSTN or shNC. (F) GO enrichment analysis was performed to identify pathways associated with the representative DEGs. (G) The bubble plot displays the top 20 markedly enriched KEGG pathways for the DEGs. (H) The mRNA expression of BMP4 in macrophages induced sEVs derived from CAF-S5/-S6 transfected with shNC and shPOSTN were analyzed by RT-qPCR. (I) The mRNA expression of TNF- α , IL-6, TGF- β and IL-10 in macrophages stimulated with BMP4 at concentrations of 0, 50 and 100 ng/ml. (J) The expression of CD163 and CD206 in macrophages treated with BMP4 at concentrations of 0, 100 ng/ml examined by flow cytometry. (K) The level of BMPR2, pSmad1/5/9, Smad 5 and Smad 9 in macrophages treated with BMP4 (100 ng/ml) for 48 h were examined by western blotting (n=3 per group). (L) The level of pSmad1/5/9, Smad5, Smad9, CD163 and CD206 in macrophages treatment with or without LDN193189 inhibitor (n=3 per group). Statistical significance was determined using a one-way ANOVA test, ns, not significant *P<0.05; **P<0.01; ****P<0.0001. Error bars represent the mean $\pm$ SEM. Source data for blotting assays, see Fig. S5. PCA, principal component analysis; sEV, small extracellular vesicles; sh, short hairpin; NC, negative control; DEGs, differentially expressed genes; POSTN, perostin; sEV, small extracellular vesicles; GSEA, gene set enrichment analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; RT-qPCR, reverse transcription-quantitative PCR; pSmad, phosphorylated Smad.

at the time of analysis and was implemented to bolster the robustness and diversity of the immune cell profiling, following rigorous batch effect correction. Importantly, the inclusion of the PBMC dataset served as a critical negative control. As expected, fibroblasts markers were absent in PBMC-derived cells. Specifically, POSTN expression was exclusively confined to fibroblasts clusters within the tumor samples and was completely undetectable across all immune cell populations.

The current study deciphered the heterogeneity of fibroblasts and identified distinct populations of POSTN⁺ and POSTN⁺ fibroblasts. Moreover, it was demonstrated that eCAF and apCAF signatures were highly expressed in POSTN⁺ fibroblasts, whereas myCAF and iCAF signatures were enriched in POSTN⁺ fibroblasts. Functionally, apCAF serve an essential role in promoting the tumor immunosuppressive environment by modulating T-cell activity (65), whereas POSTN⁺ fibroblasts suppress CD8⁺ T-cell infiltration, impair antitumor immunity and foster an immunosuppressive microenvironment, affecting the response to immunotherapy. Hence, the potential interaction between POSTN⁺ apCAF and macrophages plays a key role in shaping the immunosuppressive microenvironment of HNSCC. The epithelial-mesenchymal transition (EMT)-related gene signature serves as a novel mechanistic explanation for the underlying prognosis of hepatocellular carcinoma (HCC) (66). EMT is a crucial step in tumor invasion and metastasis and CAFs serve as the primary drivers inducing tumor EMT (66). The POSTN⁺ fibroblasts identified in the current study inherently possess a robust mesenchymal phenotype and potent ECM remodeling capacity. These POSTN⁺ apCAF may not only directly act on tumor cells by secreting EMT-inducing factors (such as TGF- β), but could also indirectly create favorable conditions for tumor cells that have undergone EMT to evade immune surveillance by constructing an immunosuppressive microenvironment. At the molecular level, future investigations should explore between the crosstalk between POSTN⁺ apCAF and established oncogenic pathways. A previous study demonstrated that CLSPN drives HCC progression via the Wnt/ β -catenin pathway (67) and existing evidence indicates that Wnt signaling is closely associated with the activation of CAFs and that β -catenin activation can regulate the expression of multiple chemokines (67). Therefore, a possible hypothesis is that aberrant Wnt signaling in tumor cells may reprogram fibroblasts into a POSTN⁺ apCAF phenotype, thereby initiating the macrophage recruitment and activation. This may represent an important research direction for linking intracellular oncogenic signaling with systemic remodeling of the TME. However, it should be noted that the use of integrated public scRNA-seq datasets may introduce inherent limitations, such as batch effects or variations in sample processing, which could affect the interpretation of fibroblast heterogeneity.

According to functional enrichment analysis, POSTN⁺ fibroblasts were primarily enriched in genes regulating collagen metabolism and ECM assembly. Although their specific mechanisms in driving ICB resistance require full elucidation, current evidence indicates that they contribute to immunotherapy resistance by upregulating PD-1 and CTLA4 protein expression on CD4⁺CD25⁺ T lymphocytes (17). Furthermore, FAP⁺ α SMA⁺ CAFs promote collagen

production, forming multi-layered physical barriers that shield tumor cells from T-cell contact (68), whereas high levels of LRRC15⁺ CAFs are associated with poor immunotherapy outcomes (69). Furthermore, the current study revealed that patients with HNSCC and high expression levels of POSTN and FAP, exhibited markedly poorer responses to ICB therapy. The co-expression of FAP and POSTN in HNSCC is markedly associated with poor prognosis, supporting their potential as prognostic biomarkers. The present study revealed that POSTN and FAP expression levels were positively associated with macrophage infiltration in TCGA-HNSCC. Further integrated analysis of TCGA-HNSCC demonstrated that POSTN^{high}/FAP^{high} HNSCC tumors exhibited characteristic immune evasion signatures and poorer ICB response rates, potentially indicating an immune-resistant phenotype. H&E and immunohistochemical (IHC) staining identified higher expression levels of FAP and POSTN in the stroma of OSCC cases compared with normal controls. However, it is important to note the inherent limitations of the IHC methodology employed. While instrumental for validating expression and spatial distribution, IHC is a semi-quantitative technique susceptible to subjective interpretation. Moreover, its static nature cannot directly demonstrate dynamic cellular interactions. For instance, although antigen-presenting fibroblasts were observed proximal to immune cells, IHC alone cannot confirm their functional role in antigen presentation or establish causality within communication networks. These functional insights warrant future investigation using techniques such as multiplex fluorescence IHC, transmission electron microscopy, co-culture models and *in vivo* loss-of-function experiments.

Several studies have investigated POSTN⁺ fibroblasts and SPPI⁺ macrophages in HNSCC. These studies have primarily reported associations between individual gene expression and patient survival, lacking deep insight into their interactions and effects on the TME (70,71). Notably, this crosstalk was investigated from multiple aspects in the current study, including single-cell transcriptomics, immunofluorescent labeling of clinical specimens and bioinformatics analysis of published datasets. In the HNSCC cohort from TCGA and in other scRNA-seq datasets, SPPI⁺ macrophages have been shown to interact with POSTN⁺ fibroblasts, with the interaction intensity being positively associated with poor overall survival (72,73). Using integrative single-cell and spatial transcriptomics, researchers have identified a significant colocalization of POSTN⁺ fibroblasts and SPPI⁺ macrophages. This finding suggests that their interaction may serve a critical role in establishing a hypoxic, TNF- α -rich microenvironment conducive to tumor progression via NF- κ B signaling (74). Furthermore, the present study highlighted the potential value of POSTN⁺ fibroblasts in HNSCC in facilitating an immunosuppressive microenvironment by inducing macrophage M2 polarization via sEV-POSTN. The robust positive association between POSTN expression and macrophage infiltration, supported by the experimental evidence that POSTN⁺ sEVs may directly steer M2 polarization through the BMP4/BMP2/Smad pathway, underscores a distinct mechanism of immune regulation. To spatially validate the interplay between POSTN⁺ fibroblasts and macrophages, spatial transcriptomics will be further employed to delineate this precise interplay in HNSCC.

The prominence of POSTN⁺ fibroblast-macrophage interactions in HNSCC is likely driven by the distinct etiology and anatomical location of the disease. The persistent exposure of head and neck mucosa to inflammatory triggers and microbiota fosters a unique microenvironment that favors dominant stromal-immune crosstalk (75). Consequently, targeting POSTN or its downstream signaling in macrophages represents a promising, disease-specific therapeutic strategy. This approach to alleviate immunosuppression and overcome ICB resistance may hold greater value in HNSCC than in malignancies where POSTN⁺ fibroblasts primarily fulfill other biological roles.

CAFs recruit monocytes and promote their differentiation into M2-like TAMs through various factors, including cytokines (CXCL12, CCL5, IL-6 and IL-33) and the glycoprotein chitinase 3-like-1 (76-78). In particular, POSTN⁺ fibroblasts attract and polarize macrophages into an SPP1⁺ TAM phenotype via the IL-6/STAT3 signaling pathway. This process hinders effective T-cell infiltration and diminishes immunotherapy response (31). Consequently, patients exhibiting high co-expression of POSTN⁺ fibroblasts and SPP1⁺ macrophages demonstrate poorer outcomes in immunotherapy cohorts (31). The present analysis of TCGA-HNSCC data revealed that the expression of POSTN and FAP was markedly positively associated with M1 macrophage markers (CD80 and CD86) and M2 macrophage markers (CD163 and CD206). This notable finding led to the hypothesis that POSTN may actively modulate M1 macrophages polarization, raising a critical question for further research. Immunofluorescence staining revealed the co-expression of POSTN and CD163 in the stromal area of HNSCC cases, suggesting a potential interaction within the TME. It should be noted that preliminary analysis was performed on four representative samples. Future studies with larger sample sizes are warranted to confirm and extend these findings.

sEVs may also be useful adjuncts to existing immunotherapeutics. Despite the profound success of ICB, a notable percentage of patients treated with these agents develop treatment resistance. PD-L1⁺ sEVs are used as a potential target, which impair anti-PD-L1 therapy by binding and targeting therapeutic antibodies, thereby enhancing their clearance by macrophages (79). It is expected that blocking PD-L1 expression or release in EVs may enhance ICB efficacy and overcome resistance in patients. Human CPC-derived exosomal vesicles carrying a short POSTN isoform (aa 22-669), have been shown to promote cardiomyocyte proliferation through activation of phosphorylation-induced activation of focal adhesion kinase, actin polymerization and nuclear translocation of Yes-associated protein signaling (36). Our previous study revealed that LOX binds the FN/POSTN/BMP4 complex located on the surface of sEVs (37). Immunogold labeling localized POSTN to the membrane surface in the present study. Furthermore, ELISA confirmed that the levels of sEV-POSTN from CAF-S5/-S6 increased in a concentration-dependent manner and this signal was markedly reduced by pretreatment with a POSTN-blocking antibody. Given the membrane localization and functional importance of sEV-POSTN, a critical next step is to investigate how POSTN knockdown alters sEVs cargoes and how such alterations subsequently influence macrophage phenotype and function.

The current study also demonstrated that CAF sEVs derived POSTN promoted the M2 macrophage polarization by activating BMP4-induced BMPR2/Smad signaling. These findings suggested that sEVs are crucial mediators of the crosstalk between CAFs and macrophages. CAFs have emerged as promising targets for anti-tumor therapy through the modulation of key signaling pathways (10). To improve chemotherapy and immunotherapy outcomes, researchers are currently investigating FAP⁺ CAF-directed therapies, including antibody-drug conjugates and CAR-T cells in preclinical and clinical studies (80,81). However, the clinical benefits of these approaches for patients with HNSCC remain unclear. The present study highlighted the role of POSTN⁺ fibroblasts in predicting immunotherapy response in HNSCC and identifies potential targets for enhancing treatment efficacy. Future work should incorporate retrospective analyses of immunotherapy-treated HNSCC cohorts and large-scale prospective trials to validate these findings. Additionally, further investigation is needed to assess the clinical feasibility of targeting POSTN⁺ fibroblast-macrophage interactions as a therapeutic strategy.

In conclusion, the present study demonstrated that POSTN⁺ fibroblasts may promote an immunosuppressive microenvironment and ICB resistance in HNSCC by secreting POSTN⁺ sEVs that drive M2 macrophages polarization via BMP4/BMPR2/Smad signaling, highlighting POSTN as a promising therapeutic potential target.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article, with additional raw data available from the corresponding author upon request. The HNSCC scRNA-seq datasets are publicly available from the GEO repository (GSE103322; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE103322>) and (GSE139324; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139324>). The analysis can be assessed R software. Gene expression data of TCGA-Head and neck squamous cell carcinoma (HNSCC) patients can be downloaded at <https://www.cbioportal.org/>. Immune infiltration levels were quantified using the single-sample gene set enrichment analysis (ssGSEA) method. The relevant

data and resources can be downloaded from <http://cis.hku.hk/TISIDB/download.php>. The raw RNA sequencing data reported in this paper have been deposited in the Genome Sequence Archive (82) in National Genomics Data Center (83), China National Center for Bioinformatics/Beijing Institute of Genomics, Chinese Academy of Sciences (*GSA-Human: HRA015291*) that are publicly accessible at <https://ngdc.cnbc.ac.cn/gsa-human/submit/hra/submit>.

Authors' contributions

XL, CZ and SC were responsible for conceptualization, visualization, funding acquisition and writing the original draft. CQ was responsible for methodology and data curation. YWa and WL were responsible for visualization, formal analysis, funding acquisition and investigation. QZ and YWu were responsible for funding acquisition, data curation and formal analysis. XL and CZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures involving human participants were approved by the Ethics Committee of Shanghai Stomatological Hospital, Fudan University (approval no. 2024-023). The use of HNSCC tissue sections was additionally approved by the same committee (approval no. 2024-005). Written informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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